71. (New) The *Shigella* of claim 70, further comprising an inactivated *Shiga*-toxin gene, inactivated by allelic exchange with a mutagenized *Shiga*-toxin gene that has been mutagenized *in vitro*, wherein said mutagenesis is other than only by means of a transposon inserted into the gene, and further, wherein said *Shigella* does not comprise an active copy of said *Shiga*-toxin gene.

72. (New) The *Shigella* of claim 70 or 71, wherein a marker gene is inserted into one or more of said mutagenized genes.

73. (New) A vaccine comprising the *Shigella* of claim 70 or 71 and a pharmaceutically acceptable vehicle.

REMARKS

Reconsideration of this application is respectfully requested.

With entry of this Amendment, claims 24-30, 32-41, and 43-73 are pending in this application. Claim 38 is withdrawn from consideration as drawn to a nonelected invention.

The amendments to claims 32-34, 39-41, and 43-45 are being made so that there is proper antecedent basis for the language used in the dependent claims, in view of the cancellation of claims 31 and 42.

Support for new claims 47 and 48 is found in the specification at page 9, line 10 to page 11, line 5.

Support for new claim 49 is found in the specification at page 8, lines 18-29.

Support for new claims 50-57 is found in previously examined claims 24-46, throughout the specification, and in particular at page 5, line 35 to page 6, line 17.

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Support for new claims 58-65 is found in previously examined claims 24-46, throughout the specification, and in particular at page 5, lines 18-33.

Support for new claims 66-73 is found in previously examined claims 24-46, throughout the specification, and in particular at page 5, line 35 to page 6, line 17, and page 5, lines 18-33.

By the term defective, applicants mean a *Shigella* that is either partially or completely ineffective for the trait described (i.e., either less effective or completely ineffective).

As described above, the amendments to the claims are supported by the specification and no new matter is added. Applicants respectfully submit that entry of this amendment is therefore proper.

Rejection of Claims 24-37 and 39-46 Under 35 U.S.C. § 103(a)

In Paper No. 44, the Office rejected all of the claims under 35 U.S.C. § 103(a) as allegedly unpatentable over Makino et al., Cell Vol. 46, pp 551-555, August 1986 ("Makino"), in view of Mills et al., Vaccine Vol. 6, pp 116-122, 1988 ("Mills"), Sekizaki et al., Infection and Immunity Vol. 55(9) pp 2208-2214, 1987 ("Sekizaki"), Naddif et al., Infection and Immunity Vol. 55 pp 1963-1969, 1987 ("Naddif"), and Ozenberger et al., J. Bacteriology Vol. 1679 pp 3638-3646, 1987 ("Ozenberger"). (Paper No. 44 at 3-5).

The Office characterizes Makino as "teach[ing] of a region on the large virulence plasmid of *Shigella* (*virG* gene/*icsA* gene) [sic] is required for cell-cell spread and is involved in the pathogenesis of *Shigella*" and that "transposon insertions into this region, and [a] mutant [thus made] may be a plausible candidate for a vaccine." (Id. at 4). The

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Office acknowledges that Makino "does not teach of inactivating the virG/icsA gene by a means other then [sic] a transposon." (Id. at 4).

The Office alleges that Mills teaches that attenuation of *Shigella* can be achieved by site directed inactivation of the toxin gene. The Office notes that Mills points out that reversion to virulence represents a possible problem in the use of such strains as vaccines.

Sekizaki is characterized by the Office as allegedly teaching methods of replacing the *Shigella* toxin gene with a mutant allele. Sekizaki's suggestion that toxin production is hazardous is also noted.

Ozenberger is characterized as teaching methods of using insertion and deletions of the siderophore gene enterobactin to impair the ability of bacteria to grow.

Finally, Nassif is characterized as allegedly teaching a *Shigella flexneri* mutant, which no longer produces the siderophore *aerobactin* and displays altered extracellular growth capacity. Statements in Nassif to the effect that such a mutant would not be expected to provide sufficient attenuation, but would certainly be considered additional security in a vaccine are also noted.

Based on these alleged teachings of the cited references, the Office contends that all claims are *prima facie* obvious as follows:

> Given that Makino et al. have generated *Shigella* strains with inactivated *icsA* gene via transposon insertions and that these strains have vaccine potential, and that transposon mutants have the potential for reversion to virulence, it would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention to have attenuated *Shigella* by inactivating genes required for bacterial invasion or *Shigella* toxin as described by Makino et al. and Sekizaki et al. and inactivation of the gene required for *aerobactin* as taught by Nassif using methods of allelic exchange and deletion

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> mutagenesis as taught by Mills, Sekizaki et al., and Ozenberger et al. for the expected benefit of developing a vaccine since as described by Sekizaki toxin production is a hazard in a vaccine [sic].

(Id. at 5).

In asserting that an alleged *prima facie* case of obviousness exists, the Office contends that the mention in Makino that transposon mutants have the potential for reversion to virulence motivates one of ordinary skill to use other methods of mutagenesis, not taught in Makino. The Office further asserts that Mills, Sekizaki, and Ozenberger provide such alternative methods, in the form of allelic exchange and deletion mutagenesis. Applicants respectfully traverse.

In order to make a proper *prima facie* case of obviousness by combining multiple references, the Office must show: (1) a suggestion or motivation to combine the reference teachings, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art; (2) a reasonable expectation of success; and (3) that the prior art references, when combined, teach or suggest all of the claim limitations. MPEP § 2142.

If all the claim limitations are not present -- either explicitly or by suggestion -- there can be no successful combination of the prior art to arrive at the claim. In the instant case, the cited references fail the all elements test with respect to the pending claims. Thus, the rejection of the claims under 35 U.S.C. § 103(a) is improper and should be withdrawn.

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The Rejected Claims are Nonobvious Over the Cited References

The pending rejected claims (24-30, 32-37, 39-41, and 43-46), and new claims 47-49, read on "[a] method for modifying a wild strain of an enteroinvasive *Shigella* to produce a modified strain of *Shigella* . . . comprising inactivating a [recited gene] . . . other than only by inactivation by means of a transposon inserted into the gene," as required by the method claims, or "[a] *Shigella* strain comprising: (a) an inactivated [recited gene], inactivated other than only by means of a transposon inserted into the gene," as required by the composition claims.

The Office contends that Makino provides a motivation to use mutagenesis methods "other than only by inactivation by means of a transposon inserted into the gene," as required by the rejected claims, and relies on Mills, Sekizaki, and Ozenberger as providing such a method. However, Mills, Sekizaki, and Ozenberger neither teach nor suggest a method of inactivating one of the genes recited in the claims, to generate a modified strain of *Shigella*, by a method other than the insertion of a transposon into that gene. These references may teach methods that can be described by the words "allelic exchange and deletion mutagenesis," as characterized by the Office. (See Paper No. 44 at 5). However, the instant claims do not read on any method of inactivating a gene by allelic exchange or deletion mutagenesis. Rather, the claims read on a specific method, where a gene is inactivated "other than only by inactivation by means of a transposon inserted into the gene." Nothing in any of the references cited that tells one of ordinary skill how to combine the teachings of Mills, Sekizaki, and Ozenberger to arrive at this limitation of the instant claims. Thus, the cited references do not render the instant claims obvious.

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Each of these references discloses a method of creating a mutant strain of bacteria, wherein a mutant allele is inserted into the genome of the bacteria to inactivate an endogenous gene (allelic exchange). However, the mutant allele that is inserted is one comprising a mutation, made "by means of a transposon inserted into the gene." Thus, the "inactivation" of the gene is accomplished by the insertion of the transposon.

Specifically, Mills describes experiments in which "[t]he *Stx*$^+$ K-12 strain was . . . mutagenized with a transposon and *Stx*$^-$ mutants generated." (Mills, page 121, left column) (emphasis added). After this mutagenesis step, "[t]he *Stx*- transposon mutant alleles containing the positive selection marker, resistance to kanamycin (Kmr), were subsequently transduced to conjugation proficient (Hfr) derivatives of E. coli K-12." (Id.) (emphasis added). After these derivatives are mated with *S. dysenteriae* 1 strains "all *Shigella* transconjugates that become resistant to kanamycin no longer produce Shiga toxin (i.e. have incorporated the *Stx*- marker)." (Id.). This is an allelic exchange event in which the *Stx*+ allele is replaced with the Stx- transposon mutant allele. Thus, Mills teaches a method to inactivate the Stx gene by means of a transposon inserted into the gene. In contrast, claims 24-30, 32-37, 39-41, 43-46, and 47-49 read on a method of inactivating a gene(s) "other than only by inactivation by means of a transposon inserted into the gene." As described above, this claimed method is not taught by Mills.

In the mutagenesis technique taught in Sekizaki "[t]he *Stx*+ *PyrF*+ *E. coli* derivative TS64 was mutagenized by Tn-mini*kan*, a transposon derived from Tn10, by infection with a lambda suicide vector containing the transposon." (Sekizaki at 2211-2212, bridging paragraph) (references omitted). The mutant alleles thus generated are then transferred to an Hfr strain of *E. coli* and subsequently transferred into *S.*

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dysenteriae 1, to generate "*Stx*- transposon insertion mutants of *S. dysenteriae* 1." (Id. at 2212, left column). This is an allelic exchange event in which the *Stx*+ allele is replaced with the *Stx*- transposon mutant allele. (Note that Sekizaki refers to the *Stx*- allele that they generated as *Stx*-1.) Thus, Sekizaki teaches a method to inactivate the *Stx* gene by means of a transposon inserted into the gene. In contrast, claims 24-49 read on a method of inactivating a gene(s) "other than only by inactivation by means of a transposon inserted into the gene." As described above, this claimed method is not taught by Sekizaki.

Similarly, Ozenberger teaches a method in which the Mini-kan and Tn1000 transposable elements are used to generate transposon insertion mutants in E. coli. (Ozenberger at 3639, right column). Next, "[p]ertinent mini-kan insertion mutations were transferred to the chromosome by homologous recombination." (Id.). Thus, Ozenberger teaches a method to inactivate a gene by means of a transposon inserted into the gene. In contrast, claims 24-30, 32-37, 39-41, 43-46, and 47-49 read on a method of inactivating a gene(s) "other than only by inactivation by means of a transposon inserted into the gene." This claimed method is not taught by Ozenberger.

So Mills, Sekizaki, and Ozenberger each discloses a method of creating a mutant strain of bacteria by insertion of a mutant allele into the genome of the bacteria (allelic exchange), wherein the mutant allele that is inserted is one in which the mutation was made "by means of a transposon inserted into the gene." In this method, the "inactivation" of the gene is accomplished by the insertion of the transposon. In contrast, the instant claims read on methods of producing modified *Shigella*, and *Shigella* made by methods, wherein the method involves "inactivating a [recited gene]. .

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. other than only by inactivation by means of a transposon inserted into the gene" (emphasis added). Clearly, neither Mills, Sekizaki, nor Ozenberger, considered either alone or together, teaches or suggests this limitation of the pending claims.

Applicants do not agree with the Office's characterization of Makino as providing motivation to use a mutagenesis method other than only by inactivation by means of a transposon inserted into the gene. However, even assuming, *arguendo*, that Makino did provide such a motivation, Mills, Sekizaki, and Ozenberger clearly do not provide such a method. Therefore, the combination of Makino with Mills, Sekizaki, and Ozenberger does not and can not render the rejected claims obvious. Rather, the hypothetical prior art disclosure of a possible motivation for the claimed invention, in the absence of a prior art disclosure of that claimed invention, is evidence of a long felt, but unmet, need in the art for that invention.

The Office further contends that *Shigella* strains can be modified by inactivating genes using the method of "deletion mutagenesis[,] as taught by Mills, Sekizaki et al., and Ozenberger et al.[,] for the expected benefit of developing a vaccine." (Paper No. 7 at 5). Applicants note that it is not clear from the context whether the Office's reference to the technique of deletion mutagenesis was intended to be directed to all three references or not. Because Ozenberger is the only reference that discloses a method of deletion mutagenesis, applicants will assume the Office is referring to Ozenberger specifically.

The method of deletion mutagenesis disclosed in Ozenberger is used to generate deletion mutations *in vitro*. In particular, the enterobactin region is placed into a recombinant plasmid and various deletion mutants are generated using restriction

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endonuclease and ligase enzymes. (Ozenberger at 3639, right column; 3640, right column to 3643, right column). Once the mutations are made, plasmids containing them are transformed into *E. coli* strain χ984 and minicells are isolated. Ozenberger cites Frazer and Curtiss 3rd, *Production, Properties and Utility of Bacterial Minicells*, Curr. Top, Microbiol. Immunol., Vol. 69, pp. 1-84 ("Frazer") (Attached hereto as Exhibit I) as describing the methodology used to create a minicell.

Frazer says that "the isolation of minicell-producing mutants [of bacterial strains] has been fortuitous, since there seems to be no general selective procedure available for obtaining minicell-producing strains." (Frazer at 62). Frazier presents a list of known minicell producing strains in Table 4. (Id.). This list does not mention any *Shigella* strain. One of ordinary skill in the art, looking to Frazer as an authoritative reference regarding the production of minicells, would assume that minicells can not be produced in *Shigella*, absent the fortuitous discovery of a minicell producing *Shigella* strain. This appears to be precisely what Ozenberger did. Rather than endeavoring to make such a fortuitous discovery, Ozenberger instead performed minicell experiments in *E. coli*. An *E. coli* strain is not a *Shigella* strain, and is therefore not within the scope of the pending claims.

Further, assuming for the moment that a minicell producing *Shigella* strain did exist, minicells are not cells. Rather, they "are small non-growing bodies produced by aberrant cell divisions at the polar ends of rod-shaped bacteria." (Frazer at 3). For this reason, "they are anucleate or DNA deficient." (Id.). Minicells are rarely produced in wild strains of bacteria, but are produced continuously during growth in certain mutant strains of bacteria. (Id.). The fact that minicells are anucleate and don't grow means

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that minicells cannot be considered to be a modified strain of *Shigella*, or a *Shigella*, as required by the pending claims.

Now stepping back for a moment, and considering the deletion mutagenesis technique of Ozenberger in isolation, and out of context of the remaining teachings of the reference, there is also no indication of how one of ordinary skill in the art would combine the *in vitro* mutagenesis technique taught in Ozenberger with the allelic exchange techniques taught in Mills, Sekizaki, and Ozenberger, to arrive at a method for "inactivating a [recited gene] . . . other than only by inactivation by means of a transposon inserted into the gene." In each of these references, the allelic exchange approach used involves the insertion of a mutant allele that is made by inserting a transposon into the gene to be mutated, followed by the selection of cells in which this insertion has occurred, and the gene of interest has been inactivated. The transposon used always comprises a sequence that encodes a product, which confers resistance to a selection agent and is essential for the selection step. In contrast, the *in vitro* deletion mutants of Ozenberger do not comprise a selection marker. Because the only way that the cited references disclose for inserting a selection marker is by inserting a transposon, there is no indication of how one of ordinary skill can use the deletion mutagenesis technique of Ozenberger to rectify the deficiencies of Mills, Sekizaki, and Ozenberger described above. Therefore, the combination of Makino with Mills, Sekizaki, and Ozenberger does not and can not render the rejected claims obvious, whether the *in vitro* deletion mutagenesis technique disclosed in Ozenberger is considered or not.

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New Claims 50-57 are Nonobvious Over the Cited References

New claims 50-57 read on methods of producing a modified strain of *Shigella*, or *Shigella* made by methods, wherein the method used comprises: (1) "inactivating a [gene] of the wild strain of *Shigella* by allelic exchange with a mutagenized [gene] that has been mutagenized *in vitro*;" (2) where the *in vitro* mutagenesis "is other than only by means of a transposon inserted into the gene." The only method of allelic exchange taught by the cited references is outside of the scope of either of these limitations. Specifically, the only method involving allelic exchange that is taught in the cited references is a method where (1) a gene of a wild strain of *Shigella*, or another type of bacteria, is inactivated by allelic exchange with a mutagenized gene that has been mutagenized *in vivo*, (2) where the *in vivo* mutagenesis is only by means of a transposon inserted into the gene. Claims 50-57 do not read on this method, so a disclosure of this method can not render these claims obvious.

New Claims 58-73 are Nonobvious Over the Cited References

New claims 58-65 and 66-73 are based on claims 24-30, 32-37, 39-41, and 43-49, and claims 50-57, respectively. Claims 58-65 and 66-73 read on methods of producing a modified strain of *Shigella*, or *Shigella* made by methods, wherein the modified *Shigella* "does not comprise an active [gene that was mutated]." To the extent that the Office may argue that the method for making minicells, as taught in Ozenberger, can be used to make a strain of *Shigella* -- which it clearly cannot, as described above -- applicants submit that a deletion mutant, as taught by Ozenberger, cannot be used to make a *Shigella* strain, which does not comprise an active copy of the mutated gene.

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Ozenberger transforms bacterial cells with recombinant plasmids that comprise a deletion mutant allele, but do not comprise a selection marker. Even if one of ordinary skill practiced this part of the method of Ozenberger, and did not continue to the production of minicells, the method does not result in the creation of a strain of bacteria that does not comprise an active copy of the gene that was mutated. Rather, such a strain would comprise both an active copy and a mutated copy, because the mutant allele of Ozenberger lacks a selection marker, and for this reason cannot be used for allelic exchange, because allelic exchange events could not be selected for in such an experiment.

This deficiency of Ozenberger can not be remedied by the disclosure of allelic exchange in the other cited references. The only method of allelic exchange that is taught in the cited references is a method where (1) a gene of a wild strain of *Shigella*, or another type of bacteria, is inactivated by allelic exchange with a mutagenized gene that has been mutagenized *in vivo*, (2) where the *in vivo* mutagenesis is only by means of a transposon inserted into the gene. Claims 58-65 and 66-73 do not read on this method, so a disclosure of this method can not render these claims obvious.

Conclusion

In view of the foregoing amendments and remarks, Applicants respectfully request the reconsideration and reexamination of this application and the timely allowance of the pending claims.

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Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

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Dated: October 9, 2001

By: Rebecca M. McNeill
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Part #49

Application Number: 08/466,698
Filing Date: June 6, 1995
Attorney Docket Number: 2356.0043-02

APPENDIX TO AMENDMENT OF OCTOBER 9, 2001

Version with Markings to Show Changes Made

Amendments to the Claims

32. (Amended) The method of [claim 31] any of claims 24-26, wherein one or more of said [mutagenized] inactivated genes are [mutagenized] inactivated genes from which at least one nucleotide sequence[s] has[ve] been deleted.

33. (Amended) The method of [claim 31] any of claims 24-26, wherein one or more of said [mutagenized] inactivated genes are [mutagenized] inactivated genes into which at least one nucleotide sequence[s] has[ve] been inserted.

34. (Amended) The method of claim 33, wherein a marker gene is inserted into one or more of said [mutagenized] inactivated genes.

39. (Amended) The *Shigella* of claim 37 [or 38], wherein the *Shiga*-toxin gene is *Shiga*-toxin A.

40. (Amended) The *Shigella* [of any] of claim[s] 36[-38] or 37, wherein said *Shigella* is S. *dysenteria* or *S. flexneri*.

41. (Amended) The *Shigella* of claim 37, [or 38] comprising [an] inactivated ent F, Fep E, Fep C, or Fep D subunit genes of the enterochelin operon.

43. (Amended) The *Shigella* of claim [42] 36 or 37, wherein one or more of said [mutagenized] inactivated genes are [mutagenized] inactivated genes from which at least one nucleotide sequence[s] has[ve] been deleted.

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44. (Amended) The *Shigella* of claim [42] 36 or 37, wherein one or more of said [mutagenized] inactivated genes are [mutagenized] inactivated genes into which at least one nucleotide sequence[s] has[ve] been inserted.

45. (Amended) The *Shigella* of claim 44, wherein a marker gene is inserted into one or more of said [mutagenized] inactivated genes.

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